Lysis of Cultured Human Melanoma M10 Cells by Polyclonal Xenoantibodies to Melanoma-associated Antigens

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ABSTRACT

Cultured human melanoma cells M10 harvested from cultures in different stages of growth show significant changes in the expression of melanoma-associated antigens (MAA), but they do not vary in susceptibility to lysis mediated by anti-MAA xenoantisera and effected by complement or lymphoid cells. Furthermore, melanoma cells M10 showed a significant increase in susceptibility to immune lysis following treatment with puromycin at doses that do not effect the expression of MAA. The lack of correlation between MAA density and susceptibility to lysis supports the contention that, under the experimental conditions used, cellular properties play a major role in the outcome of immune attack.

INTRODUCTION

Malignant transformation of cells is accompanied by functional and structural changes in their plasma membranes (for review, see Refs. 1 and 11), an event which may account for the disordered proliferation of these cells and their escape from immune attack by the host. Among such changes, the appearance of tumor-associated antigens is of interest to immunologists since these antigens participate in the interactions between malignant cells and the immune system of the host. Characterization of the expression of these antigens on tumor cells and of their relationship to immune lysis may unravel the mechanism(s) by which tumor cells avoid such lysis and suggest means of manipulating immunity to enhance destruction of tumor cells. To study this problem, we selected human melanoma cells since the immune reactions of the hosts to melanoma cells are thought to be involved in the clinical course of the disease (for review, see Refs. 5 and 7).

The results to be presented indicate: (a) that significant changes in the level of expression of MAA on the melanoma cells M10 harvested from cultures in different stages of their growth are not associated with changes in susceptibility to immune lysis mediated by operationally specific xenoantisera to MAA and effected by complement or lymphoid cells; and (b) that treatment of melanoma cells M10 with puromycin can increase their susceptibility to lysis even if the expression of MAA is not changed.

Cultured Melanoma Cells M10. These cells which express MAA (8) have been grown in suspension for over 1 year in Roswell Park Memorial Institute Medium 1640 with 10% fetal calf serum. Cells harvested by centrifugation at 130 x g for 10 min from cultures in stationary phase (when the [3H]thymidine uptake was less than 2% of the maximum) were seeded in fresh prewarmed (37°C) Roswell Park Memorial Institute Medium 1640 and incubated at 37°C. The rate of DNA synthesis and the concentration of viable cells determined in a hemocytometer by trypan blue exclusion were the criteria used to follow the growth of cultures. The extent of DNA synthesis (expressed as cpm/2 x 10^5 cells) was measured by incubating 2 ml of cell suspension with 10 µCi of [3H]thymidine at 37°C for 30 min. Then, the reaction was stopped by the addition of 10 ml of cold PBS. The cells were washed 2 times with PBS and resuspended in 2 ml of cold 5% trichloroacetic acid. The trichloroacetic acid-insoluble material was collected onto Millipore filters which were placed in counting vials containing 10 ml of scintillation fluid (Aquasol; New England Nuclear, Boston, Mass.). A Packard Tri-Carb liquid scintillation spectrometer was used for measuring [3H]thymidine.

Anti-MAA Xenoantisera. It was from a rabbit immunized with repeated intramuscular injections of cultured melanoma cells M21 and extensively absorbed with pooled human RBC, cultured lymphoid cells, and carcinoma cells. The specificity of the absorbed antisera was determined by the pattern of reactivity in a 125I-labeled SpA-binding assay with a panel of cultured human cells which included 7 melanoma cell lines, 10 B- and 2 T-lymphoid cell lines, and 4 carcinoma and 3 sarcoma cell lines. The preparation and characterization of the xenoantisera have been described in detail elsewhere (10). Fractionation of the antisera by gel filtration on Sephadex G-200 and by ion-exchange chromatography on DEAE-cellulose has shown that the antibody activity is mediated by IgG immunoglobulins.

Puromycin Treatment. Puromycin (Boehringer Mannheim, San Francisco, Calif.) at final concentrations ranging between 0.5 and 10.0 µg/ml was added to cultures of M10 cells, and the cultures were incubated at 37°C for 24 hr. Then, the cells were washed 3 times with minimum essential medium and tested for viability, expression of MAA, and susceptibility to immune lysis. The effect of puromycin on protein synthesis has been measured by assessing the incorporation of [3H]proline.

Determination of Cell Volume. A given number of M10 cells from cultures at various stages of growth were placed in cylindrical glass tubes (0.2 x 5 cm) and centrifuged at 450 x g for 10 min at 4°C. The ratio of respective cell volumes was considered equal to the height ratio of packed cells.

Serological Assays. The serological assays used in this study have been described in detail elsewhere (4, 10, 13, 14).
and therefore will be described only briefly here. The complement-dependent cytotoxic test was performed as a microtest in Moller-Coates plates; the source of complement was rabbit serum at a dilution which was not spontaneously cytotoxic to cultured melanoma cells. Cell killing was determined by the uptake of eosin (4).

In a radioimmunometric assay with $^{125}$I-labeled Staphylococcus aureus protein A ($^{125}$I-labeled SpA) (10), cultured melanoma cells ($2 \times 10^6$) were incubated with dilutions of xenonantisera (50 $\mu$l) in 96-well vinyl microtiter plates at $4^\circ$ for 30 min. After 3 washings, the cells were incubated with $^{125}$I-labeled SpA (5 $\mu$g, 10$^4$ cpm) for 30 min at $4^\circ$. Unbound $^{125}$I-labeled SpA was removed by washing, and the cell pellet was counted for $^{125}$I cpm.

In the quantitative microabsorption assay (13), the antisera at the final dilution causing total killing of target cells were absorbed with varying numbers of absorbing cells (2-fold dilutions ranging from 300 cells/$\mu$l to 50,000 cells/$\mu$l) and then tested for residual cytotoxic activity against selected target cells.

In the ADCC test (14), melanoma cells were labeled with $[^3$H]proline by incubating $2.5 \times 10^6$ cells in 2.5 ml of proline-free medium added with 1.0 $\mu$mol $[^3$H]proline (5 Ci/mmol; New England Nuclear) for 18 to 24 hr at $37^\circ$. Triplicates of 25,000 thoroughly washed target cells in 50 $\mu$l of medium were incubated for 15 min at $37^\circ$ with anti-MAA xenonantisera dilutions in microtiter plates. Then, $2.5 \times 10^5$ rat splenocytes were added to each microtiter well, and incubation was continued at $37^\circ$ for 18 hr in an atmosphere of 5% CO$_2$. After addition of 100 $\mu$l of PBS to each well, the cells were resuspended and harvested on an automated sample harvester (Mash II; Microbiological Associates, Bethesda, Md.). The filter discs were dried overnight at room temperature and counted in Econofluor (New England Nuclear). Specific lysis was determined by the formula

$$\frac{E - B}{T - B} \times 100$$

where $E$ is observed release, $B$ is release in the absence of effector cells, and $T$ is total potential release by hypotonic treatment.

RESULTS

Expression of MAA Antigens on Melanoma Cells M10 Harvested from Cultures at Different Stages of Growth. Cultured melanoma cells M10 harvested from cultures at different phases of their growth were evaluated at one time to avoid variability among experiments performed on different days. To this end, a series of sequential cultures was established, all of which arrived at a different point of the growth curve simultaneously (17, 44, 68, 92, and 121 hr of culturing correspond to lag, early log, mid-log, late-log, and stationary phases, respectively. Sequential cultures of M10 melanoma cells duplicate the growth history of continuous cultures with respect to cell concentration and DNA synthesis measured by cell count and incorporation of $[^3$H]thymidine (Chart 1).

The uptake of $^{125}$I-labeled SpA by $2 \times 10^5$ melanoma cells M10 sensitized with an excess of anti-MAA xenonantisera is lowest in the initial stage of growth with a steady increase over the entire culture growth cycle, reaching a maximum level of expression in late-log to early stationary phase (Chart 2). Melanoma cells M10 harvested from cultures in lag phase have a volume about 1.5-fold larger than that of cells from cultures in log phase; then, the cell volume shows no significant change as the culture moves to the resting phase (Chart 2). By correcting the values of the $^{125}$I-labeled SpA-binding assay for changes in volumes of cells M10 harvested from cultures in different stages of growth, one can determine the relationship between the density of MAA on the melanoma cells M10 and the growth curve of the culture. The density of MAA, as measured by uptake of $^{125}$I-labeled SpA by $2 \times 10^5$ melanoma cells M10 sensitized with an excess of MAA xenonantisera, is lowest on melanoma cells M10 harvested from cultures in log phase, increases progressively as the culture moves to log phase, reaches a maximum when the culture is in late-log phase, and decreases slightly when the culture is in stationary phase (Chart 2). Similar results were obtained with a quantitative microabsorption assay in which an anti-MAA xenonantisera was absorbed with various numbers of melanoma cells from cultures in various stages of growth, and the residual cytolytic activity was measured in a complement-dependent cytotoxic assay with melanoma cells (data not shown).

Susceptibility to Immune Lysis of Melanoma Cells M10 Harvested from Cultures at Different Stages of Growth. Melanoma cells M10 harvested from cultures at different stages of growth were tested for susceptibility to lysis mediated by anti-MAA xenonantisera and effected by rabbit complement or by lymphocytes (Chart 3). The melanoma cells M10 did not change significantly in their degree of susceptibility to immune lysis as measured by the titer of the anti-MAA xenonantisera. Therefore, in this system, susceptibility to immune lysis as indicated by the titer of the anti-MAA xenonantisera used is not...
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by antiserum and lymphoid cells: the titer of the xenonantisem was increased from 1:16 to 1:128 (Chart 4). The effect of puromycin is essentially similar on melanoma cells M10 from cultures in mid-log and stationary phases.

**DISCUSSION**

Under our experimental conditions, the efficiency of killing by polyclonal anti-MAA xenoantibodies and complement or lymphoid cells is not a function of the density of MAA since: (a) the extent of immune lysis of melanoma cells M10 harvested from cultures in different stages of their growth does not change in spite of significant variations of the density of MAA; and (b) melanoma cells M10 show a significant increase in susceptibility to the lytic action of polyclonal anti-MAA xenoantibodies in conjunction with lymphoid cells, following treatment with puromycin at concentrations and for incubation periods which do not effect the expression of MAA. Our results probably do not reflect a density of MAA on melanoma cells M10 which is in large excess over that required for immune lysis to occur since: (a) complement-dependent lysis mediated by polyclonal anti-MAA xenoantibodies occurs only in conjunction with a source of complement-containing natural antibodies to human cell surface markers (3), and (b) no change in the degree of lysis was observed even when limited amounts of polyclonal anti-MAA xenoantibodies were used in the assays. Therefore, our results support the contention that, under certain experimental conditions, cellular properties (such as distribution of antigens, susceptibility of cell membranes to immune lysis, and ability of cells to repair immune damage) play a major role in the outcome of immune attack (see Ref. 2 and 12 for review).

Melanoma cells M10 treated with puromycin under our experimental conditions did not change in their sensitivity to the lytic action of anti-MAA xenoantibodies and complement. Since the lytic process effected by lymphoid cells resembles lysis effected by complement (9), the dissimilar results obtained with ADCC and complement-dependent cytotoxicity may reflect dif-
sensitivities in sensitivity of the 2 tests. Puromycin has been shown to increase susceptibility to complement-dependent lysis of human lymphoid cells sensitized with anti-HLA antibodies (6) and of guinea pig hepatoma cells sensitized with antitumor-specific antigen antibodies (15). The different results may reflect the significantly lower doses of puromycin we used to treat melanoma cells.

Our results raise 2 points of practical interest: (a) the changes in MAA expression on melanoma cells harvested from cultures in different stages of their growth may influence the reproducibility of binding assays to detect anti-MAA antibodies unless the growth phase of the cultures from which target cells are harvested is standardized; and (b) the increase in sensitivity to immune lysis of melanoma cells after treatment with puromycin may be useful in detecting low levels of antibodies to MAA in melanoma patients.

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